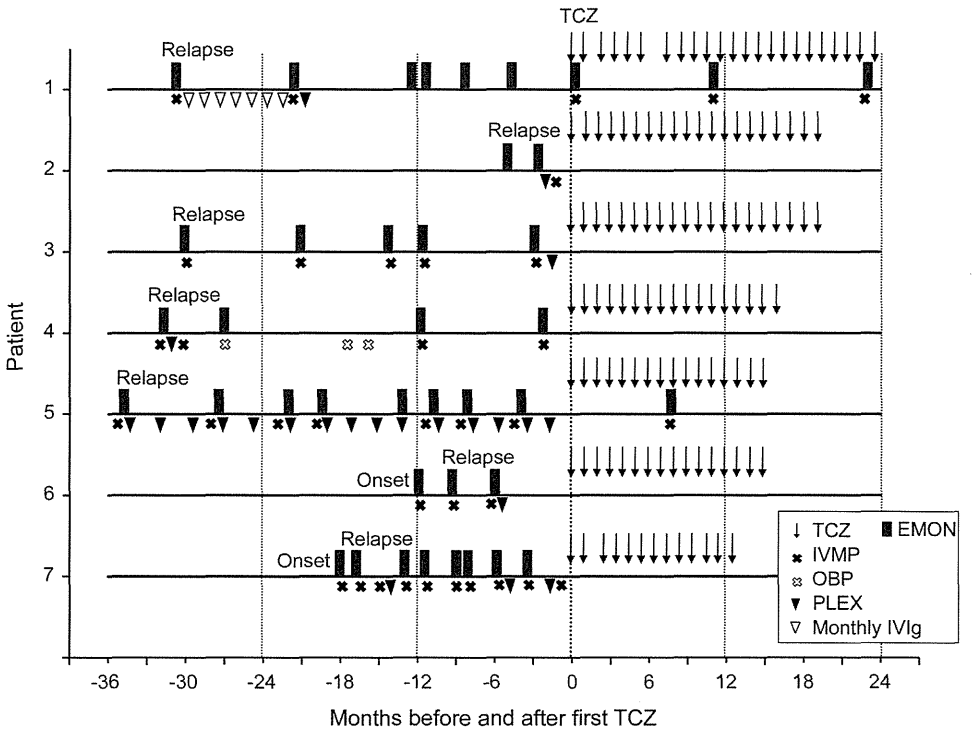
Figure 1 Clinical course of the patients before and after tocilizumab treatment



The zero on the x-axis represents the first administration of tocilizumab (TCZ). Dark gray bars: exacerbations of myelitis or optic neuritis (EMON); downward arrow: TCZ treatment; black X: IV methylprednisolone (IVMP); white X: oral betamethasone pulse (OBP) therapy; black triangle: plasma exchange (PLEX); white triangle: IV immunoglobulin (IVIg). After receiving 12 injections, all patients continued treatment with TCZ by entering an extension study that evaluates the long-term safety and efficacy of TCZ. We showed the clinical status after completion of the 1-year study to indicate the continuation of remission.

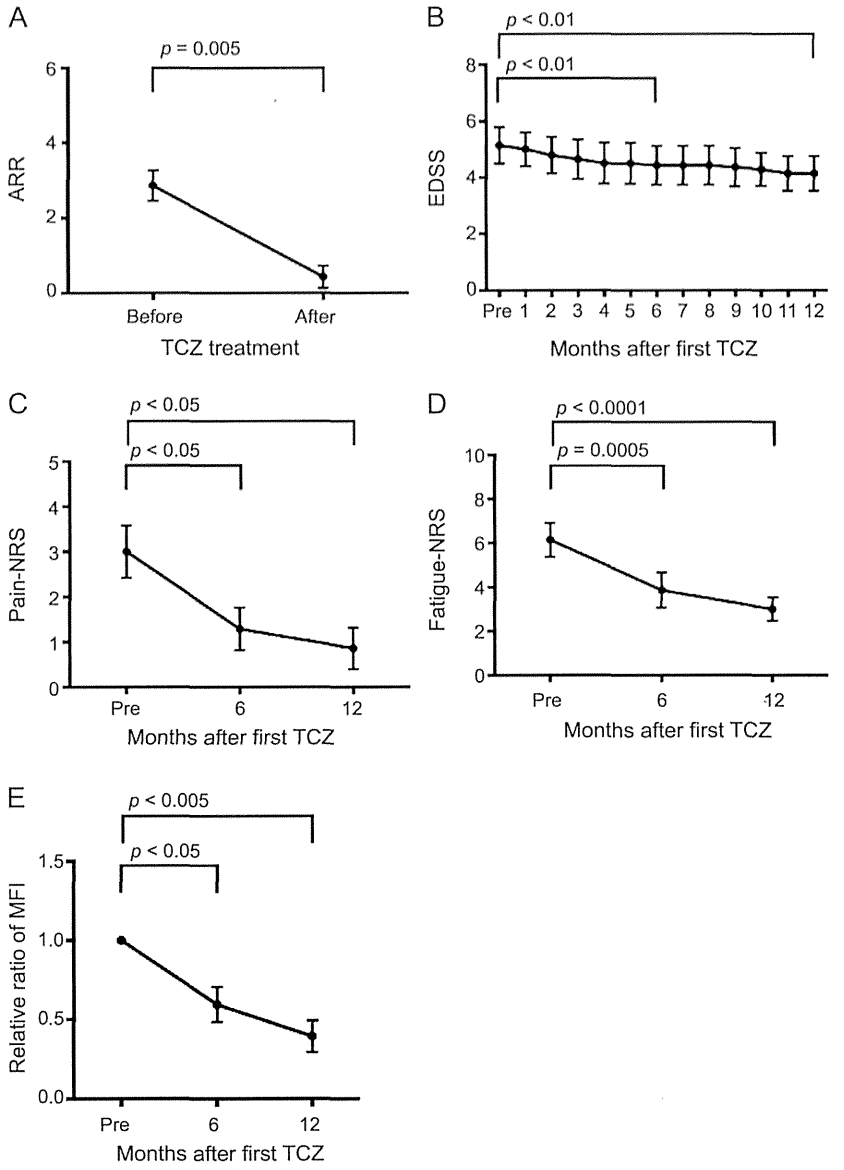
starting TCZ (figure 2). The EDSS score decreased modestly but significantly from 5.1 ± 1.7 (range, 3.0–6.5) to 4.1 ± 1.6 (range, 2.0–6.0) at 12 months. The chronic neurogenic pain in their trunk and extremities, which is characteristic of NMO[6,7] (table), gradually lessened after the patients started TCZ. Consequently, the numerical rating scale for pain reduced from 3.0 ± 1.5 upon study entry to 1.3 ± 1.3 after 6 months and 0.9 ± 1.2 after 12 months. General fatigue also improved from 6.1 ± 2.0 to 3.9 ± 2.1 at 6 months and 3.0 ± 1.4 at 12 months. The MRI scans, sensory- and visual-evoked potentials, and CSF observations did not show any interval changes. Serum anti-AQP4-Ab levels represented by the relative mean fluorescence intensity were significantly reduced (figure 2E).

Adverse events included upper respiratory infections (patients 1 and 7), acute enterocolitis (patients 1 and 4), acute pyelonephritis (patient 1), leukocytopenia and/or lymphocytopenia (patients 1, 4, and 7), anemia (patients 3 and 7), and a slight decline in systolic blood pressure (patient 1). However, none of the events was severe. Oral PSL and AZA were tapered in patients 1, 3, 4, and 7, resulting in a reduction of the mean doses (PSL from 19.5 ± 7.6 to 8.8 ± 5.6 mg/d [average of patients 1, 3, 4, and 7], AZA from 37.5 to 5.4 mg/d [average of patients 1 and 4]).

DISCUSSION Pain management is a difficult problem in patients with NMO. In fact, a retrospective study of 29 patients with NMO who experienced pain has documented that 22 of the 29 patients were taking pain medications, but none of them rated their current pain as 0 out of 10 on a 10-point scale.[6] In the present study, the intractable pain reduced gradually after the patients started TCZ treatment. After 6 or 12 months of therapy, 3 of the 6 patients with pain were completely free of pain. These results suggested a role of IL-6 in NMO pain and the possible merits of the use of TCZ in clinical practice as a pain reliever.

The pathophysiology of neurogenic pain is now understood in the context of interactions between the immune and nervous systems,[8] which involve proinflammatory cytokines such as IL-6 as well as immune cells, activated glia cells, and neurons. Supportive for the role of IL-6 in pain, recent work in

Figure 2 Effects of tocilizumab on clinical and immunologic parameters



(A) Annualized relapse rate (ARR) before and after tocilizumab (TCZ) treatment. (B) Expanded Disability Status Scale (EDSS) score during the 1-year study period. Pain severity (numerical rating scale [NRS]) (C) and fatigue severity (D) scores before, 6 months after, and 12 months after the start of TCZ treatment. The dots and I bars indicate means ± SEM. We analyzed only data obtained during the first year of TCZ treatment. (E) The alterations in the serum anti-aquaporin-4 antibody (AQP4-Ab) were evaluated by the relative ratio of the mean fluorescence intensity (MFI), which was based on the MFI before TCZ treatment. Serum anti-AQP4-Ab detection assay was performed as described previously[3,5] with minor modifications. In brief, optimally diluted serum was added to human AQP4-expressing Chinese hamster ovary (CHO) cells. CHO cell-bound anti-AQP4-Ab was detected using fluorescein isothiocyanate-anti-human immunoglobulin G antibody by flow cytometry. For comparison, the MFI of each sample was divided by the MFI of the sample before the start of TCZ treatment.

rodents showed that gp130 expressed by nociceptive neurons might have a key role in pathologic pain.[9] Although expression of membrane-bound IL-6R is restricted to hepatocytes, neutrophils, and subsets of T cells, the gp130, ubiquitously expressed in cellular membranes, can transduce IL-6R signaling via binding to the IL-6/soluble IL-6R complex.[4] This indicates that IL-6 trans-signaling via the soluble IL-6R could be pivotal in causing pain in NMO, although alternative possibilities cannot be excluded.

TCZ treatment recently showed efficacy for patients with aggressive NMO who were refractory to the anti-CD20 antibody rituximab.[10] The efficacy of TCZ could result from its effect on IL-6–dependent inflammatory

processes, involving CD20-negative PB, pathogenic T cells, and regulatory T cells. This work, however, does not restrict the use of TCZ in serious NMO. Although the need for monitoring latent infection and adverse events is obvious, we propose that the use of TCZ may be considered at an early stage of NMO before disability or a lower quality of life becomes evident.

AUTHOR CONTRIBUTIONS

T.Y., S.M., S.K., M.M., and M.A.: design and conceptualization of the study. M.A., K.M., T.O., and T.Y.: analysis and internalization of the data. T.M. and T.A.: flow cytometry analysis and anti-AQP4-Ab assay. M.A. and T.Y.: drafting and revising of the manuscript. T.Y.: supervising the entire project.

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Differential effects of fingolimod on B-cell populations in multiple sclerosis

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Abstract

Background: Fingolimod is an oral drug approved for multiple sclerosis (MS) with an ability to trap central memory T cells in secondary lymphoid tissues; however, its variable effectiveness in individual patients indicates the need to evaluate its effects on other lymphoid cells.

Objective: To clarify the effects of fingolimod on B-cell populations in patients with MS.

Methods: We analysed blood samples from 9 fingolimod-treated and 19 control patients with MS by flow cytometry, to determine the frequencies and activation states of naive B cells, memory B cells, and plasmablasts.

Results: The frequencies of each B-cell population in peripheral blood mononuclear cells (PBMC) were greatly reduced 2 weeks after starting fingolimod treatment. Detailed analysis revealed a significant reduction in activated memory B cells ($CD38^{int\text{-}high}$), particularly those expressing Ki-67, a marker of cell proliferation. Also, we noted an increased proportion of activated plasmablasts ($CD138^{+}$) among whole plasmablasts, in the patients treated with fingolimod.

Conclusions: The marked reduction of $Ki\text{-}67^{+}$ memory B cells may be directly linked with the effectiveness of fingolimod in treating MS. In contrast, the relative resistance of $CD138^{+}$ plasmablasts to fingolimod may be of relevance for understanding the differential effectiveness of fingolimod in individual patients.

Keywords

B cells, CD38, CD138, fingolimod, memory B cell, multiple sclerosis, plasmablast, proliferation, resistance, sphingosine 1-phosphate receptor 1

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Introduction

It is currently assumed that a large proportion of autoreactive T cells in multiple sclerosis (MS) is derived from a pool of $CCR7^{+}$ central memory T cells that are passing through the secondary lymphoid tissues (SLT).[1] Accordingly, egress of the T cells from the SLT represents a key process in MS pathogenesis. This process follows a rule of chemotaxis, in which the sphingosine 1-phosphate (S1P) receptor 1 (S1P1) expressed by lymphocytes is critically involved.[2] Fingolimod, an oral drug for treating relapsing–remitting MS (RRMS), serves as a functional antagonist for S1P1: Fingolimod induces internalisation and degradation of S1P1 in lymphocytes, causing the lymphocytes to lose the ability to respond to S1P and consequently, to become trapped in the SLT.[3] Analysis of large cohorts of patients with RRMS demonstrate the overall effectiveness of fingolimod in reducing the annualised relapse rate (ARR), as well as the appearance of new brain lesions in the patients' magnetic resonance imaging (MRI) scans.[4,5]

The number of central memory interleukin 17-producing $CD4^{+}$ T cells (Th17 cells) is reduced in the peripheral blood of fingolimod-treated patients. This is now being interpreted as a major mechanism of drug action;[6] however, fingolimod is not able to prevent relapses nor exhibit

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Table 1. Clinical data of the patients in this study.

Patient	Gender	Age (years)	Duration (years)	Relapse frequency (last 2 yrs)	EDSS	DMT before initiation of fingolimod	Complications
1	M	34	7	5	1.5	IFNβ1a + PSL	Asthma
2	M	43	6	2	2.5	PSL	Graves' disease
3	M	39	5	1	3.5	None	Depression
4	M	41	13	1	3.5	IFNβ1b	None
5	M	29	2	3	2.0	IFNβ1b	Pectus excavatum
6	F	41	24	6	3.5	IFNβ1b → GA → Dex	Depression
7	M	56	16	2	5.5	IFNβ1b → IFNβ1b + PSL → IFNβ1a + AZP	Osteoporosis
8	M	41	9	2	4.0	IFNβ1b → IFNβ1a	Depression
9	M	60	20	1	3.5	AZP → MZR → IFNβ1b	None
mean ± SD		42.7 ± 9.8	11.3 ± 7.4	2.5 ± 1.8	3.3 ± 1.2		

AZP: Azathioprine; Dex: dexamethasone; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; F: female; GA: glatiramer acetate; IFN: interferon; M: male; MZR: mizoribine; PSL: prednisolone.

appreciable effectiveness in all patients. In fact, recent case reports document the presence of fingolimod-treated MS patients who have developed tumefactive brain lesions, after receiving fingolimod.[7–10] Moreover, clinical worsening accompanied by large brain lesions is described in patients with neuromyelitis optica (NMO), within months of starting fingolimod.[11,12] Our current understanding of fingolimod-related biology therefore remains incomplete, particularly regarding differential effectiveness in individual patients.

Not only the presence of clonally-expanded B cells in the central nervous system (CNS),[13,14] but the efficacy of the anti-CD20 monoclonal antibody (mAb) rituximab[15] rationally indicates the involvement of B cells in the pathogenesis of MS. Therefore, B-cell migration can serve as a therapeutic target in MS, so we were prompted to investigate whether inhibition of B-cell migration may explain the differential effectiveness of fingolimod. Because the effects of fingolimod on B cells in MS have not been fully characterised,[16] we analysed the alterations of B-cell populations in fingolimod-treated RRMS patients by flow cytometry, measuring the frequencies and activation states of their peripheral blood B-cell populations.

Materials and methods

Patients and sample collection

The following subjects were enrolled in the Multiple Sclerosis Clinic of the National Centre of Neurology and Psychiatry (NCNP) in Japan:

(a) Fingolimod-naïve patients with RRMS ($n = 9$);
(b) RRMS patients who were treated with other disease-modifying treatments (DMTs) or corticosteroids ($n = 19$); and
(c) Healthy donors ($n = 3$).

All MS patients fulfilled the revised McDonald criteria.[17] Fingolimod (0.5 mg once/day) was administered to nine fingolimod-naïve patients. These patient's blood samples were collected before and 2 weeks after initiating fingolimod therapy. Most of these patients discontinued other DMTs at least 2 weeks before entry into the study, due to non-responsiveness to their DMT treatment or due to adverse events. The absence of serum anti-aquaporin 4 (AQP4)-Ab was confirmed by cell-based assays.[18,19] Upon MRI, no patient showed longitudinally-extensive spinal cord lesions extending over three or more vertebrae. The clinical data of these nine patients are summarised in Table 1.

Control blood samples were collected from 19 patients with RRMS (mean age ± SD: 41.8 ± 13.8 years; female:male ratio: 15:4) who had not been exposed to fingolimod before nor during the study. The three healthy donors were males (mean age ± SD: 40.0 ± 3.6 years). This study was approved by the Ethics Committee of the NCNP. We obtained written informed consent from all subjects.

Reagents

The following fluorescence- or biotin-labelled mAbs were used: anti-CD19-allophycocyanin (APC)-cyanine 7 (Cy7), anti-CD27-V500 and anti-CD27-phycoerythrin (PE)-Cy7 (BD Biosciences, San Jose, CA, USA); anti-CD180-PE and anti-CCR7-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Jose, CA, USA); anti-CD38-FITC, anti-CD3-FITC and mouse IgG1-FITC (Beckman Coulter, Brea, CA, USA); anti-CD138-APC, mouse IgG1κ-APC, anti-HLA-DR-Pacific Blue, mouse IgG2Aκ-Pacific Blue, anti-CD183 (CXCR3)-peridinin-chlorophyll-protein (PerCp)-cyanine 5.5 (Cy5.5), mouse IgG1κ-PerCp-Cy5.5, anti-CD38-APC, anti-CD38-PerCp-Cy5.5, anti-CD14-Pacific Blue, anti-Ki-67-Brilliant Violet, mouse IgG1κ-Brilliant Violet and streptavidin-PE-Cy7 (BioLegend, San

Diego, CA, USA); and anti-CXCR4-biotin and mouse IgG2A-biotin (R&D Systems, Minneapolis, MN, USA).

Cell preparation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Ficoll–Paque Plus (GE Healthcare Bioscience, Oakville, ON, Canada). B-cell populations were defined in reference to our previous paper,[19] as follows: total B cells, $CD19^+$; naïve B cells (nBs), $CD19^+CD27^-$; memory B cells (mBs), $CD19^+CD27^+CD180^+$; and plasmablasts (PBs), $CD19^+CD27^+CD180^-CD38^{high}$.

To evaluate the frequency and activation state of each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-V500, anti-CD38-FITC, anti-CD180-PE, anti-CD138-APC, anti-CXCR3-PerCp-Cy5.5, anti-CXCR4-biotin, streptavidin-PE-Cy7 and anti-HLA-DR-Pacific Blue. To assess the expression of CCR7 in each B cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC, anti-CD180-PE and anti-CCR7-FITC.

For examining Ki-67 expression in each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-PerCp-Cy5.5, anti-CD180-PE and anti-CD138-APC, then fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and permeabilised with 0.1% saponin. Subsequently, these cells were stained with anti-Ki-67-Brilliant Violet. We used the appropriate isotype control antibodies as negative controls for each staining. At the end of the incubation, the cells were washed and resuspended in PBS supplemented with 0.5% bovine serum albumin (BSA) and analysed by FACS Canto II (BD Biosciences), according to the manufacturer's instructions.

Cell sorting

PBMC were labelled with CD3 and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated into positive and negative fractions by AutoMACS (Miltenyi Biotec). The positive fraction was stained with anti-CD3-FITC and anti-CD14-Pacific Blue, whereas the negative fraction was stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC and anti-CD180-PE. Each positive and negative fraction was sorted into $CD3^+$ T cells and $CD14^+$ monocytes, or into nBs, mBs and PBs by a FACS Aria II cell sorter (BD Biosciences). The purity of the sorted cells was > 95%.

Quantitative real-time PCR

Messenger ribonucleic acid (mRNA) was prepared from the sorted cells using the RNeasy Kit (Qiagen, Tokyo, Japan), further treated with DNase using the RNase-Free DNase Set (Qiagen), and reverse-transcribed to complementary DNA (cDNA) using the cDNA Synthesis Kit (Takara Bio, Shiga, Japan). We performed polymerase chain reaction (PCR) using iQ SYBR Green Supermix (Takara Bio) on a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). RNA levels were normalised to endogenous β-actin (ACTB) for each sample. The following primers were used: S1P1 forward, CGAGAGCACTACGCAGTCAG; and S1P1 reverse, AGAGCCTTCACTGGCTTCAG.

Data analysis and statistics

We used Diva software (BD Biosciences) to analyse our flow cytometry data. We performed the statistical analysis with Prism software (GraphPad Software, San Diego, CA, USA). Paired or unpaired *t*-tests were used once the normality of the data was confirmed by the Kolmogorov-Smirnov test. Otherwise, the Wilcoxon signed-rank test or the Mann-Whitney *U*-test was used, as appropriate. One-way analysis of variance (ANOVA) was used to compare data from more than two groups. If the one-way ANOVA was significant, we performed *post hoc* pairwise comparisons using Tukey's test. A p value < 0.05 was considered statistically significant.

Results

B-cell populations express S1P1 mRNA

First, we used flow cytometry to examine S1P1 expression on the surfaces of the B-cell populations; however, surface S1P1 was hardly detected (data not shown). This is probably because of its internalisation following S1P binding. In support of this, it is known that S1P is abundantly present in peripheral blood.[2] Thus, we measured S1P1 mRNA in purified lymphocyte populations from the PBMCs of three healthy donors. Each B-cell population was identified by flow cytometry, as shown in Figure 1(a). We found that comparable levels of S1P1 mRNA were expressed in T cells, nBs and mBs. In comparison, PBs expressed a significantly lower level of S1P1, and S1P1 expression in monocytes was virtually absent (Figure 1(b)). Of note, a lower S1P1 expression by PBs, as compared with other B cell populations, is also described in mice.[20,21] These S1P1 mRNA expression profiles suggested that not only T cells, but B-cell migration, could also be influenced by fingolimod.

Next, we measured the frequencies of the B-cell populations in the PBMCs from nine patients with RRMS, before and 2 weeks after starting fingolimod. Results of flow cytometry showed that the frequencies of nBs, mBs and PBs among PBMCs were significantly decreased after initiating fingolimod treatment (Figure 1(c)). We confirmed that the absolute numbers of each population in the peripheral blood were also significantly decreased after starting fingolimod (Figure 1(d)). The mean decrease rate ± SD of each cell population was calculated based on the absolute cell number, giving the following results: total B cells, 87.6 ± 5.8%; nBs, 88.1 ± 6.0%; mBs, 85.4 ± 9.1% and PBs, 89.8

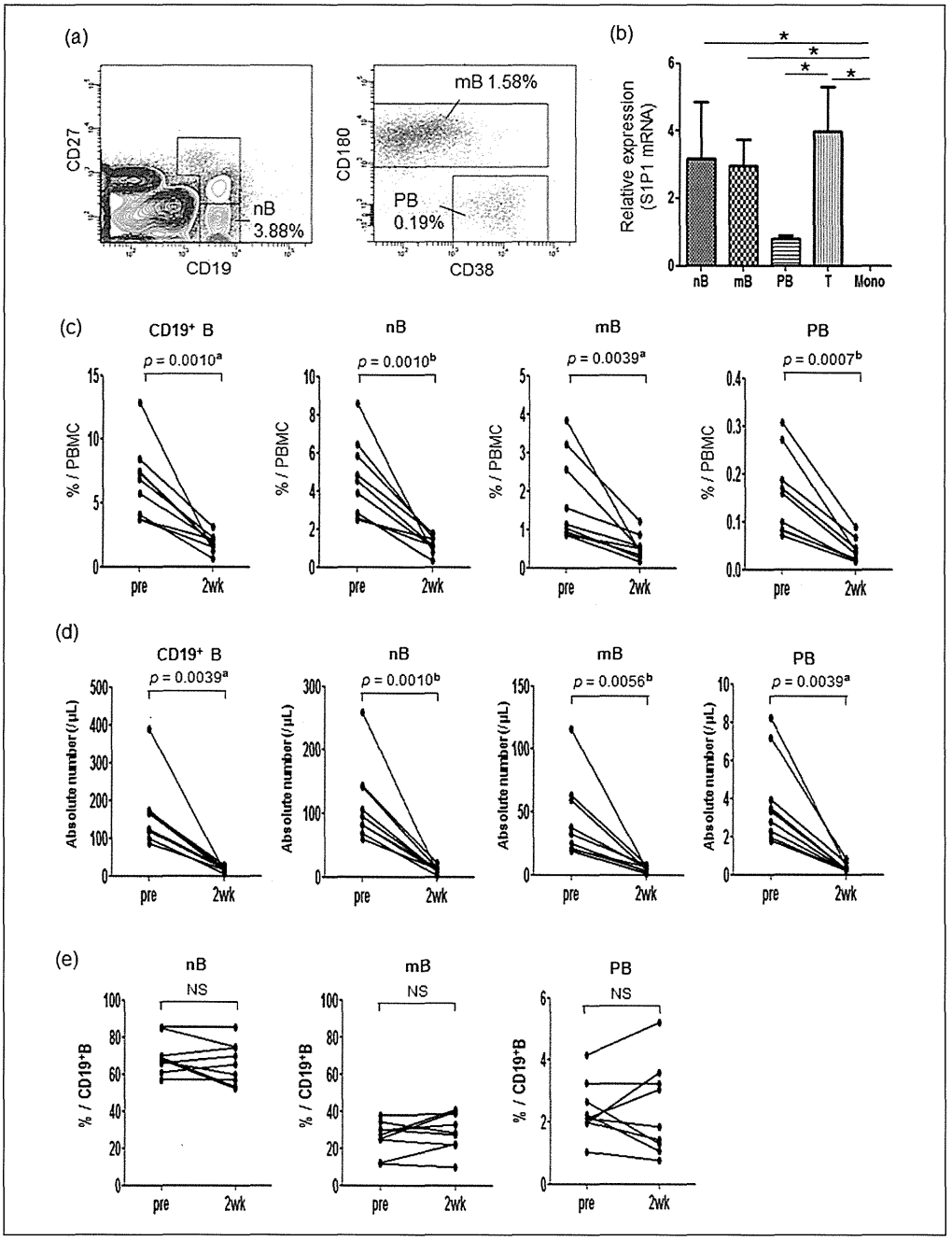

Figure 1. Frequency and absolute number of each B-cell population found in peripheral blood from MS patients.
(a)Representative flow cytometry scheme to analyse B-cell populations in PBMC. The PBMC were simultaneously stained with fluorescence-conjugated anti-CD19, -CD27, -CD38 and -CD180 mAbs. The gate for $CD19^+CD27^-$ nBs is shown in the left panel. The $CD19^+CD27^+$ fraction partitioned in the left panel was analysed for CD180 and CD38 expression to specify $CD180^+$ cells (mBs), and for $CD180^-CD38^{high}$ cells (PBs) in the right panel. Values represent frequencies of B-cell populations in PBMC. Total $CD19^+$ B cell counts were calculated by summing the frequencies of the partitioned populations in the left panel. (b) Each B-cell population, $CD3^+$ T cells and $CD14^+$ monocytes in PBMCs from three healthy donors were sorted by FACS, and S1P1 mRNA expression levels were determined by quantitative RT-PCR. Data were normalised to the amount of ACTB for each sample. Data are represented as mean relative expression ± SD. *$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c), (d), and (e) Data shown are the frequencies of B-cell populations in PBMC (c), the absolute numbers of B cell populations in peripheral blood (d) and the frequencies of B-cell populations in $CD19^+$ B cells (e) from nine patients with MS before (pre) and 2 weeks after (2 wk) initiating fingolimod. Data from the same patients are connected with lines.
$p^a < 0.05$ by Wilcoxon signed-rank test.
$p^b < 0.05$ by paired *t*-test.
ACTB: endogenous beta actin; ANOVA: analysis of variance; FACS: Fluorescence-activated cell sorting; mAbs: monoclonal antibodies; mBs: memory B cells; mono: monocytes; mRNA: messenger ribonucleic acid; MS: multiple sclerosis; nBs: naïve B cells; NS: not statistically significant; PBMC: peripheral blood mononuclear cells; PBs: plasmablasts; pre: before treatment; RT-PCR: reverse transcriptase - polymer chain reaction; S1P1: sphingosine 1 phosphate receptor 1; T: T cells; 2 wk: 2 weeks after treatment initiation.

± 3.3%. Thus, all B-cell populations decreased at similar rates, regardless of their S1P1 expression levels. We also noticed that reduction of the B-cell populations did not correlate with CCR7 expression (a large proportion of nBs and mBs expresses CCR7, whereas only a small percentage of PBs expresses CCR7 (Supplementary Figure 1)). Consistently, the frequency of each B-cell population within $CD19^+$ B cells was not significantly altered in the fingolimod-treated patients (Figure 1(e)).

$CD38^{int}$- and $CD38^{high}$-activated memory B cells are preferentially decreased in fingolimod-treated patients

We next assessed mBs, which are assumed to play an important role in MS.[22,23] To evaluate the effects of fingolimod on the activation state of mBs, we first analysed CD38 expression of mBs in the nine patients, before and after initiating fingolimod. CD38 is a marker that is upregulated upon B-cell activation.[24] We found that mBs could be classified into three subpopulations according to CD38 expression levels ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$). Notably, frequencies of $CD38^{int}$ and $CD38^{high}$ mBs were significantly decreased 2 weeks after initiating fingolimod, whereas the frequency of the $CD38^{low}$ subpopulation became significantly increased (Figure 2(a) and (b)).

We further examined the expression of another activation marker, HLA-DR, within the $CD38^{low}$, $CD38^{int}$ and $CD38^{high}$ mB subpopulations. We found that the $CD38^{high}$ subpopulation expressed a significantly higher level of HLA-DR, compared with the $CD38^{low}$ mB population, as assessed by mean fluorescence intensities (MFIs) (Figure 2(c) and (d)). Although not statistically significant, HLA-DR expression in the $CD38^{int}$ subpopulation was intermediate, compared with that in the $CD38^{low}$ mB subpopulation. We also found that the MFIs of forward scatter (FSC), which reflects cell size, were significantly higher in the $CD38^{high}$ subpopulation, compared with the $CD38^{low}$ and $CD38^{int}$ subpopulations (Figure 2(c) and (d)). These findings suggest that $CD38^{high}$ mBs may contain a larger number of recently-activated blastic cells.

Fingolimod reduced Ki-67^+ recently-activated memory B cells in peripheral blood

The nuclear antigen Ki-67 is exclusively expressed in the active stages of the cell cycle (G1, S, G2 and M phases),[25] and Ki-67^+ circulating immune cells are considered to be recently activated cells that have just egressed from the SLT. To clarify whether $CD38^{high}$ and $CD38^{int}$ mB subpopulations are enriched for recently-activated cells, we examined the frequency of Ki-67^+ cells in each mB subpopulation, in the six MS patients who were not treated with fingolimod. This analysis revealed that $CD38^{high}$ mBs contained a significantly higher frequency of Ki-67^+ cells than did $CD38^{low}$ and $CD38^{int}$ mBs, and that $CD38^{int}$ mBs were likely to contain a higher frequency of Ki-67^+ cells than the $CD38^{low}$ mBs (Figure 3(a) and (b)). In addition, we compared the frequency of Ki-67^+ cells in each mB subpopulation, between fingolimod-treated ($n = 5$) and -untreated control patients ($n = 6$), and found that $CD38^{int}$ and $CD38^{high}$ mBs of the fingolimod-treated patients contained a significantly lower percentage of Ki-67^+ cells compared with those of the untreated patients (Figure 3(c)). These findings suggest that recently activated mBs are enriched in $CD38^{int}$ and $CD38^{high}$ subpopulations and that fingolimod efficiently blocks the egress of these cells from the SLT into the peripheral circulation.

The $CD138^+$ subpopulation in plasmablasts is relatively resistant to fingolimod

Finally, we analysed alterations of PBs by fingolimod in more detail. As PBs serve as migratory B cells that produce pathogenic autoantibody directed against AQP4,[19] their role in the antibody-mediated pathology is being considered also in the pathogenesis of MS. Notably, CD138 expression appears to separate PB subpopulations that could become differentially altered during the inflammatory process. In fact, $CD138^+$ PBs have a higher potential to migrate to inflamed tissues than $CD138^-$ PBs.[26] Moreover, as has recently been reported by us, $CD138^+$HLA-DR^+ PBs are selectively enriched in the cerebrospinal fluid (CSF) during relapse of NMO, and the $CD138^+$HLA-DR^+ PBs migrating to the CSF express CXCR3.[27] Therefore, we compared the frequencies of $CD138^+$ cells in PBs, as well as their expression of HLA-DR and CXCR3, before and after fingolimod treatment.

We found that the frequencies of $CD138^+$ PBs among total PBs were significantly increased after fingolimod initiation (Figure 4(a) and (b)); however, the absolute numbers of both subpopulations decreased, implying that $CD138^+$ PBs are relatively resistant to fingolimod, compared with $CD138^-$ PBs (Supplementary Figure 2(a) and (b)). After initiating fingolimod, $CD138^-$ PBs showed lower expression of HLA-DR, whereas the percentages of $CXCR3^+$ cells remained unchanged (Figure 4(c) – (e)). In contrast, fingolimod treatment did not significantly reduce the expression level of HLA-DR among $CD138^+$ PBs. More interestingly, $CD138^+$ PBs became more enriched with $CXCR3^+$ cells after initiating fingolimod (Figure 4(c) – (e)). The definition of PBs as $CD19^+CD27^+CD180^-CD38^{high}$ cells in this study was modified to efficiently specify autoantibody-producing cells;[19] however, adopting a more commonly used definition of PBs as $CD19^+CD27^+CD38^{high}$ cells did not alter the results (Supplementary Figure 3(a) – (e)).

Discussion

Previous studies show that fingolimod markedly decreases the number of T and B cells in the peripheral blood, without

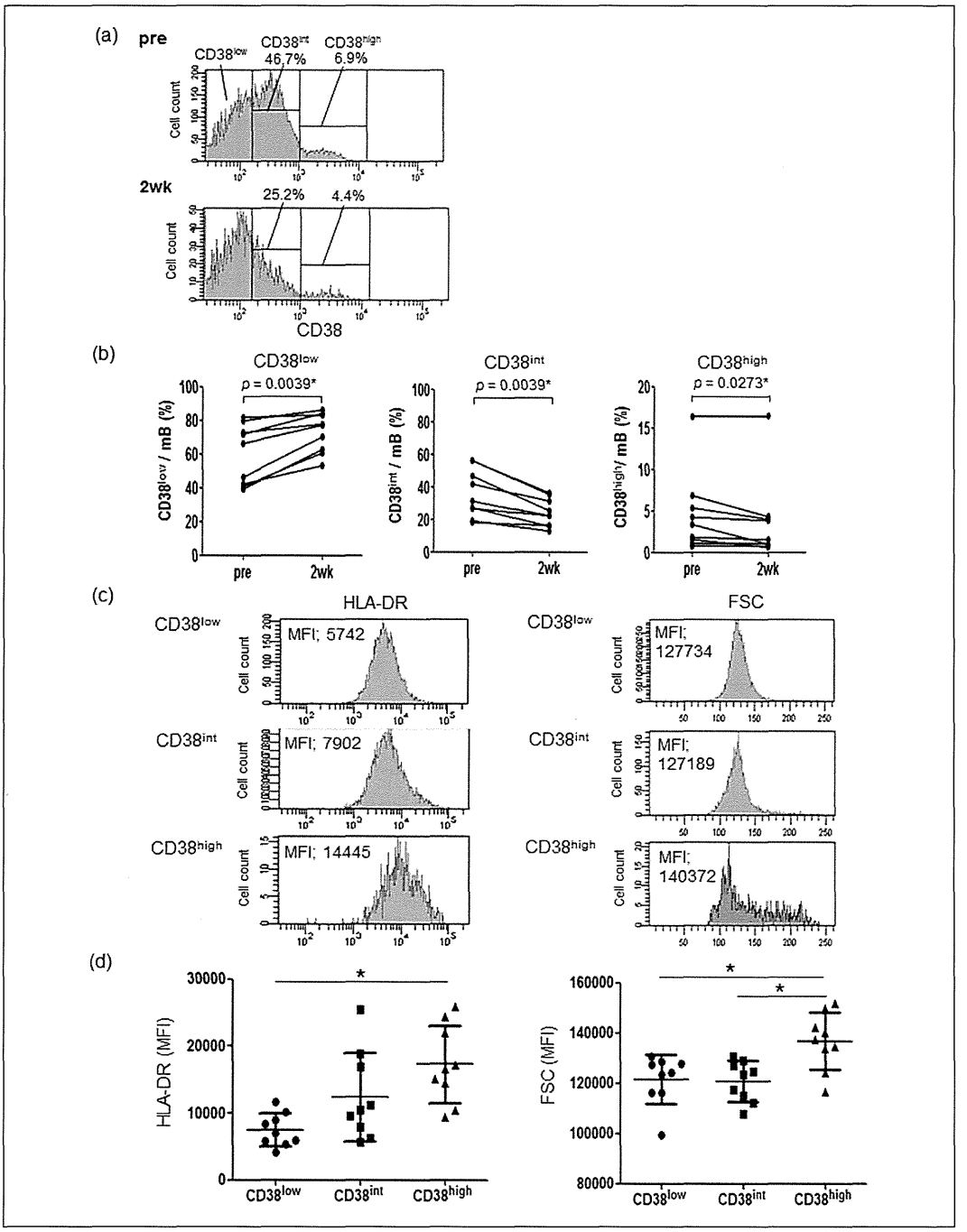

Figure 2. Frequency and activation state of each mB subpopulation in the peripheral blood of MS patients.
(a)Representative histograms of CD38 expression in mB of peripheral blood from a fingolimod-treated patient. Upper (pre) and lower (2wk) panels show the histograms before and 2 weeks after fingolimod initiation, respectively. The two values above each histogram indicate frequencies of the mB subpopulations with intermediate ($CD38^{int}$, left) and high ($CD38^{high}$, right) CD38 expression. (b) Data shown are frequencies of mB subpopulations, classified by CD38 expression levels ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel) and $CD38^{high}$ (right panel)), in the peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. $^*p < 0.05$ by Wilcoxon signed-rank test. (c) Representative histograms of HLA-DR (left column) and FSC (right column) expression in each mB subpopulation ($CD38^{low}$ (upper row), $CD38^{int}$ (middle row) and $CD38^{high}$ (lower row)) of peripheral blood from a patient with MS, before fingolimod initiation. Values represent MFIs of HLA-DR and FSC. (d) Data shown are MFI of HLA-DR (left panel) and FSC (right panel) in mB subpopulations ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$) of peripheral blood from nine patients with MS, before fingolimod treatment. Data are represented as mean ± SD.
$^*p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test.
ANOVA: analysis of variance; FSC: forward scatter; HLA: human leukocyte antigen; mB: memory B cells; MFI: mean fluorescence intensity; MS: multiple sclerosis; pre: before treatment; 2wk: 2 weeks after treatment initiation.

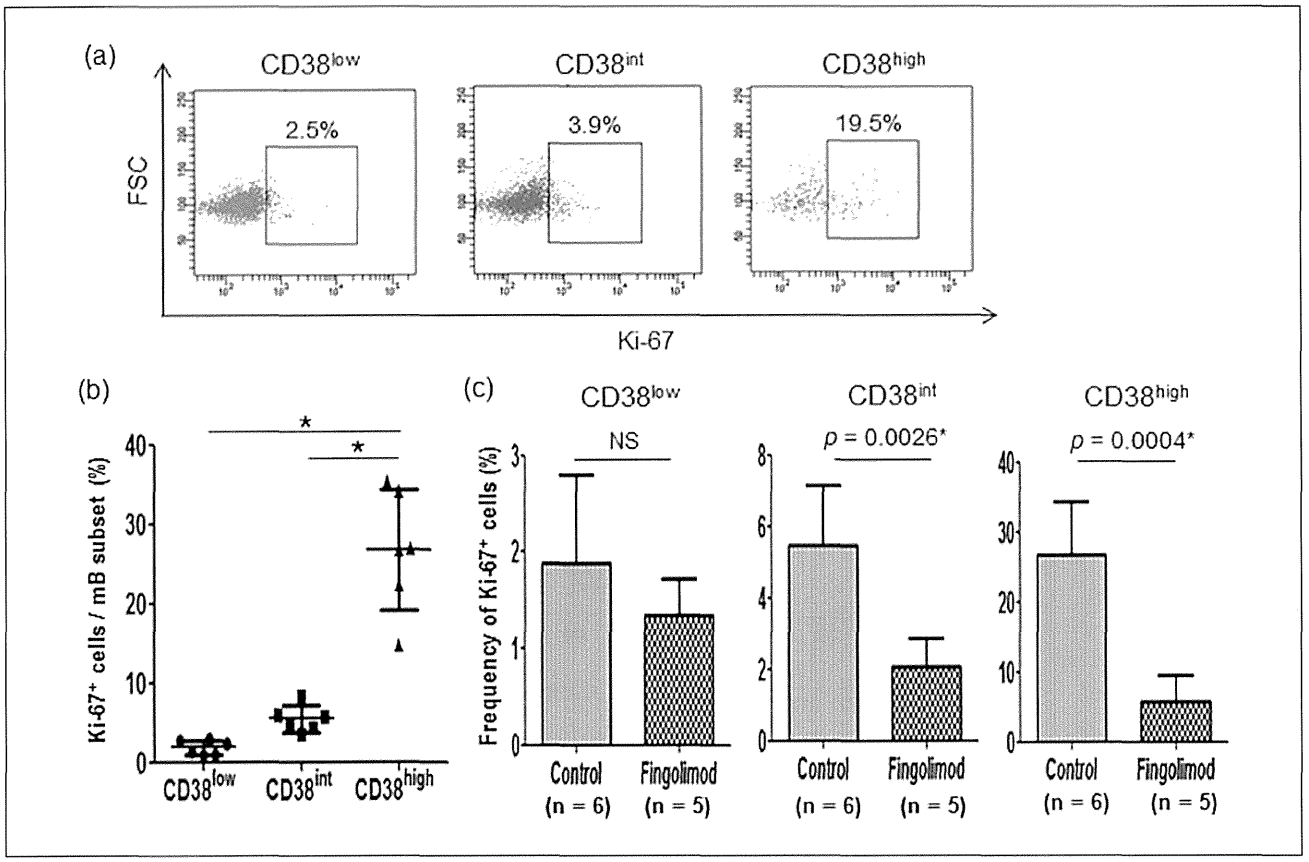

Figure 3. Ki-67 expression in mB subpopulations of peripheral blood from MS patients.
(a)Representative flow cytometry analyses of intracellular Ki-67 expression in mB subpopulations ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) of peripheral blood from an untreated patient with MS. Each mB subpopulation was analysed for FSC and Ki-67 expression. Values in each plot represent frequency of $Ki\text{-}67^+$ cells in each mB subpopulation. (b) Frequency of $Ki\text{-}67^+$ cells in each mB subpopulation of peripheral blood from six untreated patients with MS. Data are represented as mean ± SD. $^*p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c) Frequency of the $Ki\text{-}67^+$ population in each mB subpopulation ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) is compared between untreated patients with MS (control; $n = 6$) and fingolimod-treated patients with MS (Fingolimod; $n = 5$). Mean duration with fingolimod treatment ± SD is 15.8 ± 8.8 (6 to 30) weeks. Data are represented as mean ± SD.
$^*p < 0.05$ by unpaired *t*-test.
FSC: forward scatter; Ki-67: a marker present only during cell growth or proliferation; mB: memory B cells; MS: multiple sclerosis; NS: not statistically significant.

affecting the total numbers of monocytes and natural killer (NK) cells.[16,28,29] Furthermore, in MS, fingolimod selectively reduces naïve T cells, as well as $CD4^+$ central memory T cells that are enriched for Th17 cells.[6,30] In addition, fingolimod treatment may induce a relative increase in $CD27^-CD28^-$ $CD8^+$ T cells[31] and a decrease in $CD56^{bright}CD62L^+CCR7^+$ NK cells.[32]

The role of autoreactive $CD4^+$ T cells in MS pathogenesis has been emphasised over decades.[33] In contrast, B-cell involvement in MS was highlighted lately, after the clinical effectiveness of rituximab was demonstrated in RRMS patients. Rituximab's effectiveness in MS may result from the depletion of autoantibody-producing B cells, but it can also be explained by depletion of B cells that are able to induce or support activation of autoreactive T cells.[15] In fact, B cells exhibit the ability to present antigen to T cells, and mBs are more capable than nBs of supporting the proliferation of neuroantigen-specific $CD4^+$ T cells, in vitro.[23] The presence of oligoclonal bands in the CSF suggests local production of antibodies within the CNS.[34] Consistent with this, brain lesions[13] and CSF[14] of patients with MS contain clonally-expanded B cells. These results collectively support the postulate that mBs can potentially trigger the inflammation of MS, either via autoantibody production or via autoantigen presentation to autoreactive T cells.

The focus of this study is to investigate the alterations of peripheral blood B-cell types in fingolimod-treated patients with RRMS. We showed that activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were highly susceptible to

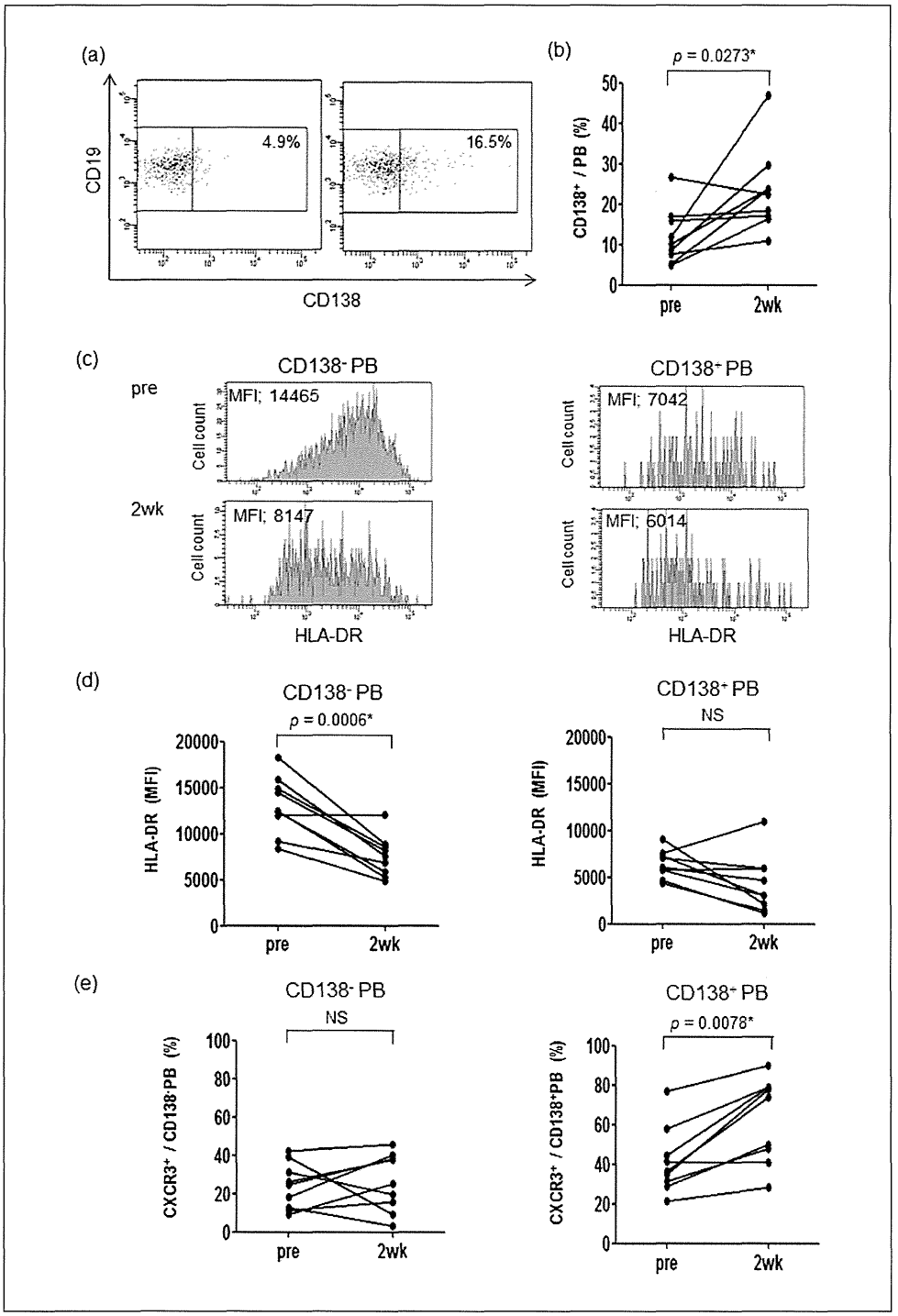

Figure 4. Phenotypic alteration of the remaining PBs in peripheral blood following fingolimod treatment.
(a)Representative dot plots of $CD19^+CD27^+CD180^-CD38^{high}$ PB, analysed for CD19 and CD138 expression before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent frequencies of the $CD138^+$ subpopulation in total PB. (b) Data are frequencies of the $CD138^+$ subpopulation in total PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test. (c) Data are representative histograms of HLA-DR expression in $CD138^-$ and $CD138^+$ PB of peripheral blood, from a patient with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent MFI of HLA-DR. (d) Data are MFI of HLA-DR in $CD138^-$ and $CD138^+$ PB of peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by paired *t*-test. (e) Data are frequencies of $CXCR3^+$ cells in $CD138^-$ PB and $CD138^+$ PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test.
MFI: mean fluorescence intensity; MS: multiple sclerosis; NS: not statistically significant; PB: plasmablast; pre: before treatment; 2wk: after 2 weeks of treatment.

fingolimod, as indicated by their reduction in the peripheral blood following fingolimod treatment. It is demonstrated in mice that surface expression levels of S1P1 on B cells in the SLT are controlled by transcription levels and CD69-mediated internalisation of S1P1. Stimulation of B-cell receptors induces not only a cessation of S1P1 transcription, but also an upregulation of CD69. Both of these changes reduce the expression levels of surface S1P1 in the SLT to some extent.[2]

Although we were not able to directly analyse B cells in the SLT of the patients, we speculated that surface S1P1 expression on mBs within the SLT in human may also decrease greatly, following antigen activation and exposure to fingolimod, which would result in these B lymphocytes having a reduced responsiveness to S1P. In fact, the activated mB subpopulations that we isolated from the patients' peripheral blood, in particular $CD38^{high}$ mB, were found to contain a substantial proportion of Ki-67^+ cells (Figure 3(a) and (b)). We confirmed that the proportions of Ki-67^+ cells in the activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were significantly decreased following fingolimod treatment, suggesting that recently-activated cells were selectively trapped in the SLT following fingolimod treatment. Because activation of autoreactive mBs in the SLT followed by their migration to the CNS could trigger a relapse of RRMS,[35] we assumed that inhibition of activated mB cell egress from the SLT was at least partly involved in the reduced relapses of RRMS after fingolimod treatment.

We also identified a PB subpopulation that is relatively resistant to fingolimod as being $CD138^+$ PBs. The frequency of the $CD138^+$ subpopulation in the total PBs, and that of $CXCR3^+$ cells in $CD138^+$ PBs, was significantly increased by fingolimod treatment. Of note, the $CD138^+CXCR3^+$ PBs are enriched in the CSF of NMO during relapse,[27] and fingolimod could induce exacerbation of NMO, accompanied by the appearance of large brain lesions.[11,12] Although knowledge on the biology of PBs is limited, the percentages of $CCR7^+$ cells are much lower as compared with nBs or mBs, indicating that fingolimod may differentially alter the in vivo migration of PBs and other B cells.

It is of relevance to note that despite reductions of circulating lymphocytes, RRMS patients receiving fingolimod may develop clinical relapses. These relapses are not always mild, but could be serious and accompany huge brain lesions.[7–10] Although the trapping of regulatory lymphocytes in the SLT[8,9] or the enrichment for $CD45RO^-CCR7^-CD8^+$ T cells in the CSF[7] is proposed as a possible mechanism for formation of tumefactive brain lesions, we were very curious to know if the increased proportion of $CD138^+$ PBs over other lymphocytes in the peripheral blood might influence the character of the CNS pathology and induce large demyelinating lesions. In fact, it was recently reported that $CD45^+CD19^+CD138^+$ PBs are relatively enriched in the CSF of fingolimod-treated MS patients,[16] raising the possibility that the dominance of $CD138^+$ PBs in the peripheral blood is preserved or even promoted in the CNS of patients with MS who develop tumefactive brain lesions[7–10] and NMO patients who deteriorate[11,12] after being treated with fingolimod. Therefore, resistance of activated PBs in fingolimod-treated patients with MS or NMO may give us a clue to understanding the individual patients' differences regarding the effectiveness of fingolimod therapy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE Open Access

Apathy/depression, but not subjective fatigue, is related with cognitive dysfunction in patients with multiple sclerosis

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Abstract

Background: Cognitive impairment could affect quality of life for patients with multiple sclerosis (MS), and cognitive function may be correlated with several factors such as depression and fatigue. This study aimed to evaluate cognitive function in Japanese patients with MS and the association between cognitive function and apathy, fatigue, and depression.

Methods: The Brief Repeatable Battery of Neuropsychological tests (BRB-N) was performed in 184 Japanese patients with MS and 163 healthy controls matched for age, gender, and education. The Apathy Scale (AS), Fatigue Questionnaire (FQ), and Beck Depression Inventory Second Edition (BDI-II) were used to evaluate apathy, fatigue, and depression, respectively. Student's t-test was used to compare MS patients and healthy controls. Correlations between two factors were assessed using the Pearson correlation test, and multiple regression analysis was used to evaluate how much each factor affected the BRB-N score.

Results: In all BRB-N tests, patients with MS scored significantly lower than controls, and the effect size of symbol digit modalities test was the highest among the 9 tests of the BRB-N. Patients with MS had higher AS ($p < 0.001$), FQ ($p < 0.0001$), and BDI-II ($p < 0.0001$) scores than controls. In patients with MS, scores on most of the BRB-N tests correlated with scores on the AS and BDI-II; however, there was little correlation between scores on the BRB-N tests and those on the FQ.

Conclusions: Cognitive function was impaired, particularly information-processing speed, and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. Despite the association between cognitive variables and depression/apathy, cognitive function was impaired beyond the effect of depression and apathy. However, subjective fatigue is not related with cognitive impairment. Taken together, this suggests that different therapeutic approaches are needed to improve subjective fatigue and cognition, and thereby quality of life, in patients with MS.

Keywords: Multiple sclerosis, Cognition, Apathy, Fatigue, Depression, Japanese

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Background

The prevalence of cognitive dysfunction in multiple sclerosis (MS) has been historically underestimated due to difficulty in detecting cognitive impairment during brief office visits without performing a formal neuropsychological assessment and a widespread belief that cognitive dysfunction occurs rarely and then only in the advanced stages of the disease [1]. However, in neuropsychological studies, 40–65% of MS patients show cognitive impairment with prominent involvement of memory, sustained attention, and information processing speed [2]. Prevalence of cognitive dysfunction in MS varies among studies depending on the type of tests used and whether the studies are based in community or clinical settings, with clinical settings showing higher rates [3]. To evaluate cognitive deficits in MS, a focused measure of cognitive abilities using the Brief Repeatable Battery of Neuropsychological tests (BRB-N) was developed [4,5]. The BRB-N was originally written in English, and has been translated into other languages including Dutch [6] and Spanish [7]. Test scores on the BRB-N are influenced by variables such as age, gender, and level of education [6,8], and the BRB-N was shown to have a sensitivity of 71% and a specificity of 94% in discriminating MS patients with and without cognitive impairment [9].

Apathy has been defined as lack of motivation not attributable to diminished level of consciousness, cognitive impairment, or emotional distress, and the three domains of apathy are considered to be "deficits in goal-directed behavior", "a decrement in goal-related thought content", and "emotional indifference with flat affect" [10]. Fatigue is a frequent complication of MS, and MS patients often report that fatigue impairs their cognitive function. However, the relation between fatigue and cognitive performance is complex and inconsistent [11]. Depression is also a common symptom of MS, and recent studies suggest that information processing speed, working memory, and executive functioning of cognitive function may indeed be affected in patients with moderate to severe depression [12].

It is important for patient management to detect cognitive impairment accurately. Further, the relationship between cognitive impairment and the emergence of neuropsychiatric disorders in patients with MS remains unclear, and apathy, fatigue, and depression have not been investigated in Japanese patients with MS. The aim of this study was to evaluate cognitive function in Japanese patients with MS, and the association between cognitive function and fatigue, apathy, and depression.

Methods

Patients with MS and healthy individuals

This study was conducted between November 2010 and March 2012 with 184 Japanese patients with MS (female/male = 135/49) diagnosed using the 2005 revised McDonald criteria [13] at 18 sites (Hiroshima City Hospital, Chiba University, Tokyo Women's Medical University Yachiyo Medical Center, Niigata University, Tokyo Women's Medical University School of Medicine, Saitama Medical School, Tohoku University Graduate School of Medicine, Kinki University School of Medicine, Asahikawa Red Cross Hospital, Yamaguchi University Graduate School of Medicine, National Center of Neurology and Psychiatry, Tokai University School of Medicine, Kyushu University, Nagasaki Kawatana Medical Center, Iwate Medical University, Juntendo University School of Medicine, Kanazawa Medical University, and Hokkaido Medical Center) in Japan. Patients with neuromyelitis optica (NMO) or NMO spectrum disorders were excluded from this study. Patients were categorized according to MS subtype: 2 had primary progressive, 167 had relapsing–remitting, and 15 had secondary progressive disease, and did not experience relapses for at least 1 month before participating in this study. The mean age of the MS patients was 39.3 years (SD. 10.1; range 18–71 years). Duration of compulsory education in Japan is 9 years and the mean duration of education excluding compulsory education in this sample was 4.92 years (SD. 1.83; range 0–9 years). The mean age at onset was 30.0 years (SD. 10.1) and the mean duration of disease was 9.3 years (SD. 7.2). The mean Expanded Disability Status Scale (EDSS) was 2.38 (SD. 2.04; range 0–8.5). Among 184 patients with MS, 109 patients received interferon β (IFNβ) as disease modifying drugs (DMDs) when they participated in this study. Twenty-five patients received other DMDs such as fingolimod and natalizumab, and 50 patients did not receive any DMDs. A total of 163 healthy controls (female/male = 119/44) participated in this study. The mean age of the healthy controls was 39.2 years (SD. 11.9; range 19–76 years). The mean duration of education excluding compulsory education was 5.15 years (SD. 2.08; range 0–13 years). Differences in sex ratio, duration of education, and age at examination between the patients and controls were not significant ($p > 0.05$). People with diseases of the central nervous system or major medical illnesses were excluded from the healthy control group. All participants had adequate vision to complete testing. The study protocol was approved by the ethics committee of each participating site, and all patients and healthy controls gave their written informed consent to participate in the study.

Battery for neuropsychological evaluation, apathy, fatigue, and depressive state

Assessment of cognitive function

For neuropsychological evaluation, patients and healthy individuals completed the BRB-N, which includes tests of verbal learning and memory (selective reminding test, SRT), visuospatial memory and learning (10/36 spatial recall test, SPART), attention, information processing,

and working memory (paced auditory serial addition test, PASAT, and symbol digit modalities test, SDMT), and verbal fluency (word list generation test, WLG). The BRB-N, which was originally written in English, was translated into Japanese and used for assessment of neuropsychological functions. The test battery was administered in the following order: SRT, SPART, SDMT, PASAT, delayed recall of the SRT (SRT-D), delayed recall of the SPART (SPART-D), and WLG. Scores derived from these tests included long-term storage (SRT-LTS), consistent long-term retrieval (SRT-CLTR), and delayed recall (SRT-D) from the SRT, immediate recall (SPART) and delayed recall (SPART-D) from the SPART, total score from the SDMT, PASAT 2-second and 3-second versions (PASAT2 and PASAT3), and total score from the WLG test.

Assessment of apathy

Apathy was measured using the Apathy Scale (AS), which is an abridged version of an apathy scale designed by Robert Marin [14], with some modifications [15]. Briefly, patients were provided with four possible answers to 14 questions: "not at all", "slightly", "some", and "a lot". Each score ranged from 0 to 42 and higher scores indicated more severe apathy [15]. The AS was translated into Japanese and had been used previously in a study of Japanese patients with stroke [16].

Assessment of fatigue

In 1989, Krupp et al. reported data of fatigue in MS using the Fatigue Severity Scale [17]. The group expanded the scale of the Fatigue Questionnaire (FQ) and administered the FQ to a large group of medical and psychiatric patients [18]. The FQ, which was translated into Japanese and has been used previously [19], was used to measure fatigue in patients with MS. The FQ consists of 29 items each of which is a statement about fatigue and is rated from 1 representing "completely disagree" to 7 representing "completely agree", with a higher score indicating more fatigue [18]. Mean scores were calculated for each patient.

Assessment of depression

The Beck Depression Inventory second edition (BDI-II), which consists of 21 items rated on a scale from 0 to 3, is a valid and reliable measure of depressive state [20]. The Japanese version of the BDI-II, which was developed to be able to assess depressive symptoms in Japanese people, is psychometrically robust [21], and was used for evaluation of depression in the present study.

Statistical analysis

Statistical analyses were performed using the SAS 9.3 software package (SAS Institute Inc., Cary, NC). For analysis, raw data for the 9 tests (SRT-LTS, SRT-CLTR, SRT-D, SPART, SPART-D, SDMT, PASAT3, PASAT2, and WLG) were used, and scores for each of these tests were standardized as a mean score of 0 and standard deviation of 1. Student's t-test was used to compare average data between MS patients and healthy controls or between MS patients who received IFNβ and those who did not receive IFNβ. Correlations between two factors were assessed using the Pearson correlation test. Multiple regression analysis was used to evaluate how much each factor—patient, AS score, FQ score, and BDI-II score—affected the BRB-N score. p values less than 0.05 were considered statistically significant.

Results

BRB-N data in MS patients and healthy controls

Cronbach's alpha coefficients for all 9 BRB-N test scores were 0.93 in MS patients and 0.82 in the healthy control group, suggesting a high level of confidence. Thus, the BRB-N translated into Japanese showed a high internal consistency for each category and all scores. Table 1 shows mean BRB-N scores in MS patients and healthy controls. Table 2 shows a significant negative correlation between age at examination and each of the BRB-N components, except for WLG, was found in healthy controls. Negative correlations between duration of education and SRT-LTS, SRT-CTLR, SRT-D, SDMT, and PASAT2 were found in healthy controls, although there were no correlations between score and duration of education in the other 4 tests. In all 9 tests, scores were significantly lower in MS patients than in healthy controls. Table 2 also shows the standardized scores for each test in patients and healthy controls. To evaluate which test score is most different between patients and healthy controls, effect size (Cohen's d) was calculated. It was found that SDMT had the greatest effect size (1.34) of the 9 items (SRT-LTS, 0.67; SRT-CLTR, 0.72; SRT-D, 0.67; SPART, 0.86; SPART-D, 0.67; PASAT3, 0.95; PASAT2, 0.96; and WLG, 0.95). In the comparison of MS patients who received IFNβ and those who did not receive IFNβ, there were not any significant differences in all 9 BRB-N tests between the two groups.

Correlation of disease duration or EDSS with BRB-N in MS patients

Table 3 shows that in each of the 9 tests except the WLG, a significant but weak negative correlation was found between disease duration and score. On the other hand, relatively strong negative correlations were found between the EDSS and BRB-N scores in MS patients.

Apathy, fatigue, and depression in MS patients and healthy controls

Mean scores on the AS, FQ, and BDI-II in MS patients were 14.38 ± 6.98 (range, 0–34), 3.89 ± 1.18 (range,

Table 1 Mean BRB-N scores in patients with MS and healthy controls

BRB-N	MS patients		Healthy controls	
	Raw scores	Standardized scores	Raw scores	Standardized scores
SRT-LTS	40.85 ± 17.18 (0–72)	−0.30 ± 1.10	50.75 ± 11.68 (14–70)	0.34 ± 0.75
SRT-CLTR	31.43 ± 18.68 (0–72)	−0.32 ± 1.04	43.60 ± 14.58 (2–70)	0.36 ± 0.81
SRT-D	7.99 ± 3.07 (0–12)	−0.30 ± 1.13	9.71 ± 1.87 (5–12)	0.34 ± 0.69
SPART	18.91 ± 5.51 (5–30)	−0.37 ± 1.00	23.26 ± 4.55 (10–30)	0.42 ± 0.83
SPART-D	6.85 ± 2.34 (0–10)	−0.30 ± 1.05	8.26 ± 1.85 (1–12)	0.34 ± 0.83
SDMT	46.20 ± 15.30 (4–84)	−0.52 ± 0.94	64.30 ± 11.24 (37–91)	0.59 ± 0.69
PASAT3	40.83 ± 15.44 (0–60)	−0.40 ± 1.14	52.45 ± 7.26 (24–60)	0.45 ± 0.54
PASAT2	30.18 ± 14.02 (0–60)	−0.41 ± 1.06	41.55 ± 8.94 (18–60)	0.46 ± 0.68
WLG	21.95 ± 7.21 (2–37)	−0.40 ± 1.02	27.99 ± 5.29 (12–40)	0.45 ± 0.75

For each test, data are expressed as mean ± standard deviation scores (ranges). MS patient scores were significantly different from healthy control scores for all tests ($p < 0.0001$).

1.00–7.24), and 13.54 ± 9.32 (range, 0–45), respectively. Corresponding scores for healthy controls were 12.03 ± 5.55 (range, 0–27), 3.40 ± 0.89 (range, 1.00–5.41), and 9.47 ± 6.59 (range, 0–27). For all 3 instruments, MS patients scored significantly higher compared to healthy controls ($p = 0.0007$, $p < 0.0001$, and $p < 0.0001$, respectively), suggesting the presence of more apathy, more fatigue, and more depression in patients. In MS patients, AS, FQ, and BDI-II scores were not associated with disease duration. On the other hand, positive correlations were noted between scores on the AS, FQ, or BDI-II and the EDSS in MS patients ($\gamma = 0.17$, $p < 0.05$; $\gamma = 0.15$, $p < 0.05$; and $\gamma = 0.20$, $p < 0.01$; respectively).

Relationship between cognitive performance and measures of apathy, fatigue, and depression

Next we evaluated whether apathy, fatigue, and depression were correlated with the BRB-N. Table 4 shows that in healthy controls, AS and FQ scores were not correlated with BRB-N scores. However, SRT-LTS, SRT-CLTR, SDMT, PASAT3, and PASAT2 scores were correlated with BDI-II score. On the other hand, in patients with MS, most test scores of the BRB-N were correlated with the scores on the AS and BDI-II. However, FQ score was not correlated with any of the BRB-N tests except WLG.

Effect of patient, apathy, fatigue, and depression in the BRB-N

To examine how much each of the patient, apathy, fatigue, and depression factors affect the BRB-N score, multiple regression analysis was conducted with these 4 factors as explanatory variables for each BRB-N test. In this analysis, "patient" was defined as 1 and "healthy control" as 0. It was found that only "patient" had a significant effect in all tests, indicating that differences in BRB-N scores between MS patients and healthy controls remained significant even after controlling for the effects of apathy, fatigue, and depression (Table 5).

Table 2 Correlation between age at examination or duration of education and the BRB-N

	Age at examination				Duration of education			
	MS patients		Healthy controls		MS patients		Healthy controls	
BRB-N	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value
SRT-LTS	−0.23	0.0017	−0.53	<0.0001	0.21	0.0045	0.22	0.0055
SRT-CLTR	−0.25	0.0006	−0.55	<0.0001	0.17	0.0214	0.19	0.0155
SRT-D	−0.16	0.0318	−0.53	<0.0001	0.17	0.0199	0.21	0.0084
SPART	−0.22	0.0023	−0.32	<0.0001	0.11	n.s.	0.07	n.s.
SPART-D	−0.24	0.0009	−0.25	0.0011	0.07	n.s.	0.06	n.s.
SDMT	−0.24	0.0012	−0.44	<0.0001	0.12	n.s.	0.23	0.0027
PASAT3	−0.13	n.s.	−0.25	0.0014	0.13	n.s.	0.15	n.s.
PASAT2	−0.10	n.s.	−0.31	<0.0001	0.12	n.s.	0.19	0.0150
WLG	−0.10	n.s.	−0.11	n.s.	0.04	n.s.	−0.09	n.s.

n.s.: not significant ($p > 0.05$).

Table 3 Correlation between disease duration or EDSS score and BRB-N test scores in patients with MS

	Disease duration		EDSS	
BRB-N	γ	*p* value	γ	*p* value
SRT-LTS	−0.16	0.0271	−0.37	<0.0001
SRT-CLTR	−0.19	0.0093	−0.34	<0.0001
SRT-D	−0.18	0.0120	−0.37	<0.0001
SPART	−0.22	0.0023	−0.25	0.0005
SPART-D	−0.24	0.0010	−0.28	0.0002
SDMT	−0.18	0.0133	−0.49	<0.0001
PASAT3	−0.24	0.0012	−0.42	<0.0001
PASAT2	−0.18	0.0141	−0.40	<0.0001
WLG	−0.09	n.s.	−0.33	<0.0001

n.s.: not significant ($p > 0.05$).

Discussion

Some degree of cognitive impairment is found in at least half of all patients with MS, and cognitive impairment typically consists of domain-specific deficits rather than global cognitive decline [9,22]. Cognitive impairment may be affected by environmental and educational factors, and there have been no large population studies on cognitive function in Japanese patients with MS. The BRB-N is now widely accepted for use in clinical studies [23] as well as in clinical practice [7]. Furthermore, studies in several populations using the BRB-N have revealed that the battery is largely unaffected by language or cultural differences, thereby validating its use in different populations [6,7,24]. The values obtained from the healthy control group in our study were similar to those found in Dutch [6], Italian [24], and Spanish [7] populations, indicating that our Japanese version did not influence performance on the test.

PASAT is a complex task and its performance largely depends on information-processing speed and working memory, which are two important and separate cognitive processes involved in the execution of the test [25]. Although the PASAT involves a larger number of cognitive processes, the SDMT could provide a better index of information-processing speed, which seems to be more frequently impaired in patients with MS [25,26]. Further, SDMT is a good test to predict cognitive impairment in patients with MS, even in the early stages of the disease [27]. Our data demonstrate that cognitive function is impaired also in Japanese patients with MS, especially in terms of information-processing speed and attentional deficits, as shown by their SDMT and PASAT scores.

Previous studies demonstrated that physical disability evaluated by EDSS score was independently associated with cognitive impairment evaluated by the BRB-N [7,24,26]. We also demonstrated a correlation between physical disability and cognitive impairment in the present Japanese MS population. These data suggest that inhibition of relapses and improved prognosis with disease-modifying therapies will also benefit cognitive function.

Some previous studies suggested that cognitive performance does not seem to correlate significantly with disease duration [22,24]; however, longitudinal studies suggest that cognitively impaired patients experience ongoing cognitive decline [1,28]. The reason for these conflicting results remains unclear, although the proportion of patients with different MS subtypes (primary progressive, relapsing–remitting, and secondary progressive) or patient age may be important. Previous studies suggest that long-term treatment with IFNβ may protect against cognitive impairment in patients with MS [29,30]. In our study, there were not any significant differences in all 9 BRB-N tests between MS patients who received IFNβ and those who did not receive IFNβ, however, the durations of IFNβ treatment were various. It is difficult to conclude effects of IFNβ treatment on cognitive function

Table 4 Correlation between apathy (apathy scale), fatigue (fatigue questionnaire), and depression (BDI-II) and the BRB-N

	Apathy				Fatigue				Depression			
	MS patients		Healthy controls		MS patients		Healthy controls		MS patients		Healthy controls	
BRB-N	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value
SRT-LTS	−0.23	0.0018	−0.04	n.s.	0.05	n.s.	0.02	n.s.	−0.18	0.0208	−0.18	0.0226
SRT-CLTR	−0.22	0.0031	−0.04	n.s.	0.04	n.s.	0.02	n.s.	−0.13	n.s.	−0.16	0.0370
SRT-D	−0.23	0.0014	0.00	n.s.	0.05	n.s.	0.01	n.s.	−0.14	n.s.	−0.11	n.s.
SPART	−0.27	0.0003	−0.00	n.s.	−0.02	n.s.	−0.09	n.s.	−0.18	0.0185	−0.02	n.s.
SPART-D	−0.33	<0.0001	−0.03	n.s.	−0.01	n.s.	−0.04	n.s.	−0.16	0.0446	−0.08	n.s.
SDMT	−0.28	0.0002	−0.07	n.s.	−0.03	n.s.	−0.01	n.s.	−0.28	0.0002	−0.29	0.0002
PASAT3	−0.22	0.0033	0.12	n.s.	−0.04	n.s.	−0.06	n.s.	−0.25	0.0013	−0.21	0.0083
PASAT2	−0.21	0.0047	0.01	n.s.	−0.04	n.s.	−0.14	n.s.	−0.23	0.0031	−0.29	0.0002
WLG	−0.23	0.0016	−0.07	n.s.	0.16	0.03	0.03	n.s.	−0.15	0.0458	−0.15	n.s.

n.s.: not significant ($p > 0.05$).

Table 5 Effect of patient, apathy, fatigue, and depression factors in the BRB-N tests

	SRT-LTS		SRT-CLTR		SRT-D		SPART		SPART-D	
Explanatory variable	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**
Patient	−0.3135	<0.0001	−0.3465	<0.0001	−0.3189	<0.0001	−0.3591	<0.0001	−0.2706	<0.0001
Apathy	−0.1249	0.0314	−0.1154	0.0470	−0.1379	0.0191	−0.1107	n.s.	−0.1807	0.0025
Fatigue	0.1713	0.0035	0.1362	0.0199	0.1245	0.0352	0.0326	n.s.	0.060	n.s.
Depression	−0.1916	0.0028	−0.1405	0.0281	−0.1190	n.s.	−0.0803	n.s.	−0.0667	n.s.
Adjusted R-squared	0.1684		0.1668		0.1485		0.1659		0.1243	
	SDMT		**PASAT3**		**PASAT2**		**WLG**			
Explanatory variable	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**		
Patient	−0.5251	<0.0001	−0.3823	<0.0001	−0.3858	<0.0001	−0.4319	<0.0001		
Apathy	−0.0764	n.s.	−0.0176	n.s.	−0.0108	n.s.	−0.1511	0.0058		
Fatigue	0.1340	0.0074	0.0842	n.s.	0.0673	n.s.	0.2171	<0.0001		
Depression	−0.2664	<0.0001	−0.2471	<0.0001	−0.2516	<0.0001	−0.1708	0.0046		
Adjusted R-squared	0.3933		0.2221		0.2301		0.2647			

n.s.: not significant ($p > 0.05$).

in MS from our study, and further studies are needed about effects of DMDs on cognitive function.

In the present study, we aimed to evaluate correlations between cognitive impairment and the three factors of apathy, fatigue, and depression in MS patients. Our results demonstrate that MS patients had more apathy, more fatigue, and more depression compared with healthy controls, and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. Despite the association between cognitive variables and depression/apathy, cognitive function was impaired beyond the effect of depression and apathy. No associations between disease duration and scores on the AS, FQ, or BDI-II were found although positive correlations between EDSS and all 3 scores were found in MS patients. Other studies also demonstrated no significant longitudinal change in the Fatigue Severity Scale across a 2- to 3-year interval in patients with MS [31], and fatigue was not correlated with disease duration [32]. Together these previous and the present findings suggest that disease duration may have little association with subjective fatigue.

Apathy is one of the major neuropsychiatric symptoms in patients with MS [33]. Figved et al. reported that apathy was significantly associated with intrusions in patients with MS [34], although few studies have explored the relationship between cognitive impairment and apathy. We demonstrated impaired apathy in Japanese patients with MS compared to healthy controls, and a negative correlation was found between apathy and cognitive function. Future studies of cognitive function should also focus on apathy.

Fatigue is a common symptom of MS, and patients with MS often report a correlation between self-reported fatigue and their perception of poor performance on cognitive tests [35]. However, no relationship has been reported between fatigue and cognitive impairment [33,36]. Our results support these findings, and subjective fatigue may not be strongly associated with cognitive impairment in MS patients. However, differences exist between subjective and objective cognitive fatigue [37]. Furthermore, fatigue could lead to unemployment in MS patients and thus a reduction in quality of life [38], and it is therefore important to investigate cognitive function and subjective fatigue using different approaches.

The prevalence of major depression in patients with MS is relatively high [39] and this may affect cognitive function. Indeed, it was reported that depression influences cognitive performance [40], although in another study depression it was not found to correlate with cognitive function [41]. Despite previous inconsistent findings regarding the association between depression and cognitive function, our results demonstrated that depression was correlated with the individual tests of the BRB-N. BDI-II is an instrument to measure the severity of depression, not to diagnose major depressive disorder. Our data of BDI-II demonstrated MS patients scored significantly higher compared to healthy controls, and suggested that MS patients may suffer from sub-depressive conditions.

Conclusions

Cognitive function, in particular information-processing speed, was impaired and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. However, subjective fatigue was not associated with cognitive dysfunction. Both fatigue and cognition affect quality of life for patients with MS, and

we may need to consider therapeutic intervention to improve fatigue and cognition using different approaches.

Competing interests

MN has received funding for travel and/or speaker honoraria from Biogen Idec, Bayer Schering Pharma, and Novartis Pharma, and has served on the scientific advisory boards for Biogen Idec. T. Kohriyama has received speaker honoraria from Biogen Idec, Bayer Yakuhin Ltd., and Novartis Pharma. IK has received funding for travel and/or speaker honoraria from Novartis Pharma, Biogen Idec, and Bayer Schering Pharma. YS has received honoraria for speaking from Bayer Yakuhin Ltd., and has received personal compensation for consulting services from Biogen Idec, Teijin Pharma and Novartis Pharma. HF has received funding for travel and/or speaker honoraria from Biogen Idec, Daiichi Sankyo Inc., Dainippon Sumitomo Pharma and Novartis Pharma. IN has served on the scientific advisory boards for Biogen Idec, Novartis Pharma; received funding for trips and speaks from Bayer Yakuhin Ltd., Biogen Idec, Tanabe Mitsubishi Pharma, Novartis Pharma, and received grant support from Mitsubishi Chemical Medience Corporation. S. Kusunoki has received speaker honoraria from Teijin, Nihon Pharmaceutical, Benesis, Japan Blood Products Organization, Novartis Pharma, Asahi Kasei, and Sanofi Aventis. KN has received funding for travel and/or speaker honoraria from Biogen Idec, Bayer Yakuhin Ltd., Mitsubishi Tanabe Pharma, Nihon Pharmaceutical Co., Ltd., Teijin Pharma Ltd., and Novartis Pharma. TY has served on scientific advisory boards for Biogen Idec and Chugai Pharmaceutical Co., Ltd.; has received research support from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Teva Pharmaceutical K.K., Mitsubishi Tanabe Pharma, and Asahi Kasei Kuraray Medical CO., Ltd; has received speaker honoraria from Novartis Pharma, Nihon Pharmaceutical Co., Ltd., Santen Pharmaceutical Co., Ltd., Abbot Japan Co., Ltd., Eisai Co., Ltd., Biogen Idec, Dainippon Sumitomo Pharma Co., Ltd., Mitsubishi Tanabe Pharma, Bayer Holding Ltd., and Astellas Pharma Inc. JK is a consultant for Biogen Idec, and has received honoraria from Bayer Healthcare and funding for a trip from Bayer Healthcare and Biogen Idec. M. Matsui is part of a scientific advisory board for Biogen Idec, and has received speaker honoraria from Bayer Healthcare, Biogen Idec, and Tanabe Mitsubishi Pharma. YM has received speaker honoraria and research material from Novartis Pharma. S. Kikuchi has received speaker honoraria from Novartis Pharma, Boehringer Ingelheim, Kyowa Hakko Kirin, Dainippon Sumitomo Pharma, and FP Pharmaceutical Corporation, and serves on the scientific advisory board for Novartis Pharma. NM, M. Mori, TO, KM, K. Yoshida, T. Kanda, FY, SN, and K. Yokoyama declare that they have no competing interests.

Authors' contributions

MN was responsible for study design, data collection, and manuscript preparation. NM was responsible for statistical analysis and manuscript preparation. IK and KM were responsible for study design, data collection, and manuscript review. S. Kusunoki and S. Kikuchi were responsible for study design and manuscript review. T. Kohriyama, M. Mori, TO, YS, HF, IN, K. Yoshida, T. Kanda, KN, TY, FY, JK, SN, K. Yokoyama, M. Matsui, and YM were responsible for data collection at their respective institutions and manuscript review. All authors read and approved the final manuscript.

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